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Effects of a Nitrate-Free Interval on Tolerance, Vasoconstrictor Sensitivity and Vascular Superoxide Production

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OBJECTIVES In the present study, we tested whether a nitrate-free interval is able to prevent increases in vascular superoxide ($O_2^{\bullet -}$) and the development of hypersensitivity to vasoconstrictors and whether this may result in restoration of vascular nitroglycerin (NTG) sensitivity.

BACKGROUND Intermittent NTG-patch treatment (12 h patch on/patch off) has been shown to increase ischemic periods in patients with stable coronary arteries, suggesting a rebound-like situation during the patch-off period. Recently, we demonstrated that long-term treatment with NTG induces tolerance, which was in part related to increases in vascular $O_2^{\bullet -}$ and increased vasoconstrictor sensitivity.

METHODS New Zealand white rabbits received a continuous application of NTG patches (0.4 mg/h) or an intermittent application of NTG patches (12 h patch on, 12 h patch off) for three days. Isometric tension studies were performed with aortic rings, and vascular $O_2^{\bullet -}$ was estimated using lucigenin-derived chemiluminescence (5 μmol/liter). Expression of the copper/zinc (Cu/Zn) superoxide dismutase (SOD) was assessed by Western blotting, and SOD activity was measured by autooxidation of 6-hydroxydopamine.

RESULTS Continuous treatment with NTG caused tolerance to NTG, cross-tolerance to the endothelium-dependent vasodilator acetylcholine, increased vascular $O_2^{\bullet -}$, reduced Cu/Zn SOD expression and increased sensitivity to vasoconstrictors such as phenylephrine, serotonin and angiotensin II. On/off treatment with NTG improved tolerance, corrected endothelial dysfunction and decreased vascular $O_2^{\bullet -}$. In addition the reduction in SOD expression was less pronounced, whereas increases in the sensitivity to vasoconstrictors such as phenylephrine and serotonin remained nearly unchanged.

CONCLUSIONS Enhanced vasoconstrictor sensitivity may explain, at least in part, the rebound phenomena observed in patients during a 12-h NTG patch-off period. (J Am Coll Cardiol 2000;36: 628–34) © 2000 by the American College of Cardiology

The therapeutic benefit of the organic nitrates is limited by the development of tolerance shortly after the onset of treatment (1). Intermittent administration of patches, allowing for an 8- to 12-h nitrate-free interval, has been demonstrated to avoid tolerance (2–4), with the disadvantage of lacking protection during this period. Another potential problem of the nitrate-free period can be the development of rebound ischemia. In patients with stable angina pectoris, Freedman et al. (5) have shown an increase in the duration of silent ischemia in the nitroglycerin (NTG)-treated group as compared with the placebo group. The authors suggested that their observations may be compatible with rebound ischemia during the nitrate-free period. These data go along with recent results demonstrating a decreased anginal threshold after NTG patch removal (6,7). Using an experimental model of nitrate tolerance, we have previously shown that continuous NTG treatment for three days led to a marked attenuation of the vasodilator potency and efficacy of NTG and to endothelial dysfunction associated with increased vascular superoxide ($O_2^{\bullet -}$) levels. This is due to activation of $O_2^{\bullet -}$-producing enzymes (8–10) and decreased activity and expression of the radical scavenger enzyme copper/zinc (Cu/Zn) superoxide dismutase (SOD) (11). We have also demonstrated an increased sensitivity of the nitrate-tolerant vasculature to vasoconstrictors such as phenylephrine, serotonin and angiotensin II (12). Similarly, NTG treatment induced enhanced vasoconstrictor responses to vasoconstrictor stimuli, such as angiotensin II and phenylephrine, in the forearm circulation (13) and coronary vasoconstriction in response to the endothelium-dependent vasodilator acetylcholine (ACh). This suggests that NTG-induced changes in vasoconstrictor sensitivity may indeed represent a clinically relevant phenomenon.

On the basis of these considerations, the present study was designed to study the effects of 12-h patch on/patch off NTG treatment on the sensitivity of the vasculature to endothelium-dependent and -independent vasodilators, on vasoconstrictor sensitivity and on vascular $O_2^{\bullet -}$ production in a well-characterized animal model of nitrate tolerance. In addition, we tested to what extent a 12-h nitrate-free interval was sufficient to restore the reduced vascular SOD activity and Cu/Zn SOD expression.

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Abbreviations and Acronyms

ACh	= acetylcholine
Cu/Zn	= copper/zinc
EC_{50}	= 50% effective concentration
6-HDOPA	= 6-hydroxydopamine
LDCL	= lucigenin-derived chemiluminescence
L-NMA	= N^G-methyl-L-arginine
NOS III	= endothelial nitric oxide synthase
NTG	= nitroglycerin
$O_2^{\bullet-}$	= superoxide
SOD	= superoxide dismutase

METHODS

Animal model. New Zealand White rabbits of either gender (weight 3 to 6 kg) were studied. A region on the dorsal thorax or between the scapulae was shaved and a NTG patch was applied to the skin. The NTG patch was changed every morning. The rabbits were studied after three days of continuous NTG therapy (n = 15) or after a three-day period of 12-h patch on/patch off NTG administration (n = 18). In these animals, the last patch was removed 12 h before experimentation. Control rabbits (n = 16) received no NTG. On the morning of the study day, an intravenous injection of 1,000 U heparin was administered, followed by a lethal dose of pentobarbital. The chest was then rapidly opened, and the descending thoracic aorta was removed.

Vessel preparation and organ chamber experiments. The aorta was placed in chilled Krebs buffer and cleaned of excessive adventitial tissue. Eight 5-mm rings of thoracic aorta were suspended in individual organ chambers (25 ml) filled with carbogen-equilibrated Krebs buffer of the following composition (mmol/liter): NaCl 118.3, KCl 4.69, $CaCl_2$ 1.87, $MgSO_4$ 1.20, K_2HPO_4 1.03, $NaHCO_3$ 25.0 and glucose 11.1 (pH 7.40). During the next hour, the rest tension was increased to optimize contractions to KCl (80 mmol/liter), as described. This optimal state, occurred at 5 *g* rest tension for both NTG-tolerant and control aortic rings. Control and tolerant rings were then preconstricted with phenylephrine (0.1 to 0.3 μmol/liter). When the tone had reached a stable plateau, NTG or ACh was applied to the organ baths in cumulative concentrations (1 nmol/liter to 3 μmol/liter in semilogarithmic concentration steps), and relaxant responses were recorded continuously.

Concentration–response curves were generated for phenylephrine, angiotensin II and serotonin and quantified as the percentage of contractions to a maximally depolarizing concentration (80 mmol/liter) of KCl, as described previously (13).

Measurement of superoxide production in endothelium-intact vascular rings. Superoxide production in endothelium-intact aortic rings from control and NTG-treated animals was measured using lucigenin-derived chemiluminescence (LDCL). The details of this method have been reported previously (14). Briefly, after preparation, the rings were equilibrated for 30 min at 37°C in modified Krebs buffer at pH 7.4, containing 20 mmol/liter of N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES). Scintillation vials containing 700 μl of Krebs/HEPES buffer with lucigenin (5 μmol/liter) were placed into a scintillation counter switched to the out-of-coincidence mode. After 15 min, background counts were recorded, and a vascular ring was then added to the vial. Scintillation counts were recorded after an additional 15 min, and the respective background counts were subtracted. The vessels were then dried for 24 h at 90°C, allowed to cool and weighed.

To address the influence of endothelial nitric oxide synthase (NOS III)-derived nitric oxide (NO) on the vascular LDCL signal, the vessels were incubated for 30 min at 37°C with N^G-methyl-L-arginine (L-NMA, 1 mmol/liter), as described recently (14).

SOD activity assay. To assess the SOD activity of control and tolerant aortic rings, they were homogenized (10% wt/vol) in 50 mmol/liter KH_2PO4 and 0.05 mmol/liter EDTA (pH 6.5), as described (11). The homogenates were cleaned of debris by centrifugation at 10,000 × *g* for 20 min. Thereafter, SOD activity was assessed in the supernatants by measuring the rate of SOD-sensitive autooxidation of 6-hydroxydopamine (6-HDOPA). Autooxidation of 6-HDOPA is catalyzed by $O_2^{\bullet-}$, yielding a red adrenochrome (λ_{max} 490 nm), and is inhibited by SOD in a concentration-dependent fashion. The aortic extracts (10 to 100 μg protein in 0.9 ml homogenization buffer) were incubated at room temperature in plastic cuvettes. The reactions were started by addition of 6-HDOPA (final concentration 0.2 mmol/liter; 0.1 ml of 2-mmol/liter stock solution dissolved in N_2-gassed distilled water). The increase in absorbance at 490 nm was continuously monitored for 3 min in a double-beam spectrophotometer. In the absence of tissue extracts, the absorbance increased by 0.10 ± 0.03 U/min due to spontaneous oxidation of 6-HDOPA. This rate was decreased in the presence of known amounts of pure Cu/Zn SOD (from bovine erythrocytes, Sigma, Deisenhofen, Germany). An apparently linear concentration–rate relation was observed from 0.1 to 0.5 U SOD.

Western blot analysis. Rat aortic tissue was homogenized in ice-cold homogenization buffer containing (in mmol/liter) NaCl 99.01, KCl 4.69, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, K_2HPO_4 1.03, Na-HEPES 20, D-glucose 11.1, aprotinin 0.0015, leupeptin 0.0107, pepstatin 0.0102 and PMSF 0.0028, using a glas/glas homogenizer. The homogenate was centrifuged at 3,000 × g for 5 min to remove insoluble material. A total of 15 μg protein was then subjected to sodium dodecyl sulfate-PAGE and transferred to nitrocellulose membranes (Bio-Rad, München, Germany). Immunoblotting was performed for 2 h at room temperature with a polyclonal sheep antibody to human

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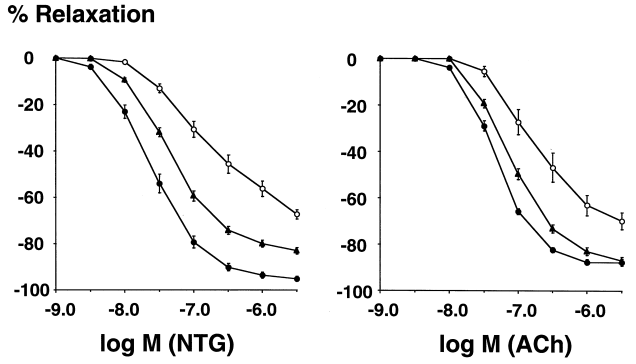

Figure 1. Effects of three-day in vivo NTG treatment of rabbits (continuous or 12 h patch on/patch off) on concentration–relaxation curves generated in vitro with NTG and ACh. Data are presented as the mean values ± SEM of six to 10 independent experiments. **Solid circles** = no treatment; **open circles** = continuous NTG treatment; and **solid triangles** = 12-h patch-on/patch-off NTG treatment.

erythrocyte Cu/Zn SOD (dilution 1:3000, Transduction Laboratories, Lexington, Kentucky). Immunodetection was accomplished with an antisheep secondary antibody (1:2000 dilution, Upstate Biotechnology, Lake Placid, New York) and the enhanced chemiluminescence kit (Amersham, Buckinghamshire, United Kingdom). Quantification of the 16-kDa Cu/Zn SOD subunits was performed by densitometry, as described recently (11). The signals were integrated, and the results expressed as percent of the control signals.

Materials. All chemicals were purchased from Sigma.

Statistical analyses. Results are expressed as the mean value ± SEM. The 50% effective concentration (EC_{50}) value for each experiment was obtained by logit transformation. To compare $O_2^{\bullet-}$ production, SOD activity and SOD expression in vessels from control animals and animals treated with continuous and intermittent NTG, one-way analysis of variance was employed. Comparisons of vascular responses were performed using multivariate analysis of variance, with animal treatment as the independent variables and percent relaxation and EC_{50} as the dependent variables. The Scheffé post hoc test was used to examine differences between groups when significance ($p < 0.05$) was indicated.

RESULTS

Effects of three-day continuous or intermittent NTG administration in vivo on the in vitro vasorelaxation to NTG and ACh. As previously demonstrated (6), continuous in vivo treatment for three days with NTG patches markedly impaired both the potency and efficacy of NTG to produce vasorelaxation in subsequent organ chamber studies (Fig. 1, Table 1). This impairment was less pronounced in the aortas from animals treated with a nitrate-free interval. Also, the potency and efficacy of the endothelium-dependent vasodilator ACh were impaired in vessels from NTG-treated animals ($p < 0.05$) (Fig. 1, Table 1). A nitrate-free interval almost completely prevented the development of endothelial dysfunction (Fig. 1, Table 1).

Effects of three-day NTG continuous or intermittent NTG administration in vivo on the in vitro sensitivity to vasoconstrictors. As shown previously, treatment of rabbits for three days with NTG caused a significant increase in sensitivity to vasoconstrictors such as angiotensin II, phenylephrine, serotonin and potassium chloride (13). In animals treated with a nitrate-free interval, the hypersensitivity to angiotensin II and partially to KCl was corrected, whereas constrictions to phenylephrine were unchanged. Interestingly, constrictions to serotonin were increased (Fig. 2, Table 2).

Effects of a nitrate-free interval on NTG-induced increases in vascular superoxide production. As demonstrated previously (6), $O_2^{\bullet-}$ production (estimated with LDCL) was increased approximately twofold in endothelium-intact aortic rings from nitrate-treated animals as compared with those from untreated animals (Fig. 3). A nitrate-free interval significantly reduced vascular $O_2^{\bullet-}$ steady-state levels, which, however, were still higher than those of control vessels.

Effects of a nitrate-free interval on NOS-mediated superoxide production. Incubation of vessels from control animals with the inhibitor of the NOS L-NMA (10^{-3} mol/liter) markedly increased the lucigenin signal, demonstrating that endothelial-derived NO markedly quenches

Table 1. Effects of Three-Day Nitroglycerin Treatment of Rabbits (Continuous or 12 h Patch On/Patch Off) on the Potency and Efficacy of Nitroglycerin or Acetylcholine to Produce Relaxations in Aortic Rings

	Potency EC_{50} (−log mmol/liter)		Efficacy (% Maximal Relaxation)	
In Vivo Pretreatment	NTG	ACh	NTG	ACh
None (control)	7.63 ± 0.08	7.32 ± 0.03	95 ± 2	87 ± 2
Continuous NTG	6.72 ± 0.12*	6.77 ± 0.11*	68 ± 4*	70 ± 4*
12-h Patch-on/patch-off NTG	7.40 ± 0.04	7.15 ± 0.11	84 ± 2*†	85 ± 3

*$p < 0.05$ vs. control group. †$p < 0.05$ vs. control group. Each value (mean ± SEM) has been calculated from five to nine experiments.

ACh = acetylcholine; EC_{50} = 50% effective concentration; NTG = nitroglycerin.

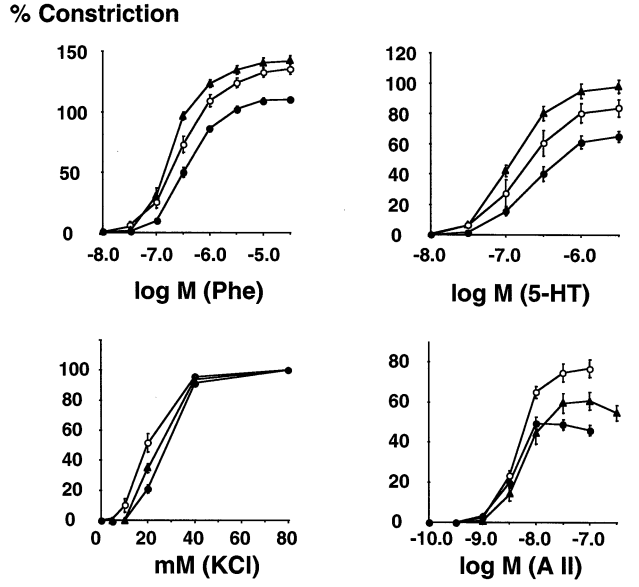

Figure 2. Effects of three-day NTG treatment of rabbits (continuous or 12 h patch on/patch off) on concentration–constriction curves generated in vitro with angiotensin II (Ang II), phenylephrine (Phe), serotonin (5-HT) and potassium chloride (KCl). Data are presented as the mean values ± SEM of 10 to 12 experiments. See Figure 1 legend for explanation of symbols.

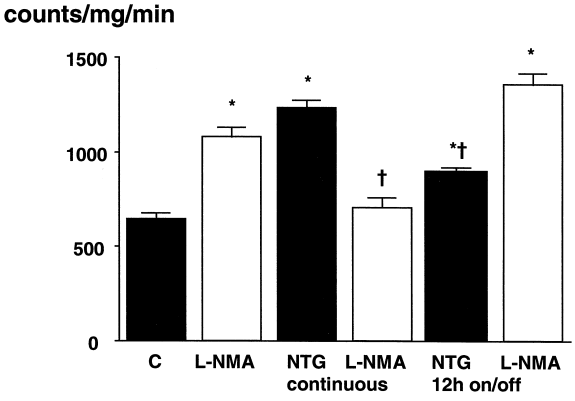

Figure 3. Effects of three-day NTG treatment of rabbits (continuous or 12 h patch on/patch off) on vascular $O_2^{\bullet-}$ production of rabbit aortic tissue with an intact endothelium. Superoxide production was measured under control conditions (C) and after the administration of L-NMA (10^{-3} mol/liter, 30-min incubation time). In vessels from control animals, L-NMA increased vascular steady-state $O_2^{\bullet-}$ levels, indicating that NO produced in endothelial cells quenches baseline $O_2^{\bullet-}$-induced LDCL. In nitrate-tolerant tissue, basal $O_2^{\bullet-}$ production was markedly elevated. Here, L-NMA paradoxically decreased LDCL, indicating that NOS III represents a significant $O_2^{\bullet-}$ source. A nitrate-free interval reduced $O_2^{\bullet-}$ levels and restored the LDCL-increasing effect of L-NMA, indicating that intermittent therapy can prevent the uncoupling of NOS III. Data are presented as the mean values ± SEM of five to 10 experiments. * Indicates $p < 0.05$ versus C; † indicates $p < 0.05$ versus NTG continuous.

the lucigenin signal (14). In the setting of tolerance, the basal lucigenin signal was increased twofold, as compared to control vessels. Preincubation of these tolerant vessels with L-NMA paradoxically decreased LDCL, indicating that in the setting of tolerance, NOS III may represent a significant $O_2^{\bullet-}$ source. In animals treated with a nitrate-free interval, the basal lucigenin signal was still somewhat increased, but L-NMA produced increases in LDCL (as in control vessels). This indicates that the 12-h nitrate-free interval is sufficient to restore the function of NOS III as an NO-producing enzyme (rather than an $O_2^{\bullet-}$-producing enzyme) (Fig. 3).

Effects of a nitrate-free interval on total vascular SOD activity and on the expression of Cu/Zn SOD. Vascular SOD activity, as assessed by inhibition of 6-HDOPA autooxidation, averaged 7.45 ± 0.3 U/mg protein in tissue homogenates from control aortic rings (Fig. 4). In vivo treatment with NTG reduced SOD activity significantly (Fig. 4). In animals treated with a nitrate-free interval, SOD activity was almost completely normalized. Likewise, in vivo treatment with NTG significantly decreased SOD expression, which was corrected by a nitrate-free interval (Fig. 5).

DISCUSSION

The present study demonstrates that a 12-h nitrate-free interval partially restores the sensitivity of the vasculature to NTG. The improved response to NTG is associated with a reduction in vascular $O_2^{\bullet-}$ production secondary to a restoration of total vascular SOD activity and Cu/Zn SOD expression. The enhanced vasoconstriction to serotonin and phenylephrine of vessels from animals treated with a nitrate-free interval may explain, at least in part, the rebound phenomena observed during the 12-h patch-off period in patients with stable coronary artery disease.

Mechanisms underlying nitrate tolerance. The mechanisms underlying nitrate tolerance are likely to be multifactorial and may involve neurohumoral activation (15) and increases in vascular $O_2^{\bullet-}$ production (9), as well as increased sensitivity to vasoconstrictors (13,16). Recent ani-

Table 2. Effects of Continuous or Intermittent Nitrate Therapy on 50% Effective Concentration and Maximal Constrictions to Phenylephrine, Serotonin, Angiotensin II and Potassium Chloride

	Potency (EC_{50})				Maximal Constriction (%)			
	Phe (−log mol/liter)	5-HT (−log mol/liter)	Ang II (−log mol/liter)	KCl (mmol)	Phe	5-HT	Ang II	KCl
Control	6.40 ± 0.05	6.62 ± 0.06	8.48 ± 0.05	27.7	109 ± 2	62 ± 7	48 ± 2	100
NTG	7.25 ± 0.11*	6.76 ± 0.11	8.34 ± 0.04	18.1*	136 ± 5*	83 ± 5*	76 ± 5*	100
NTG on/off	6.78 ± 0.11	6.85 ± 0.03	8.18 ± 0.07	24.6	140 ± 4*	98 ± 4*†	59 ± 5	100

The sensitivities of phenylephrine (PE), angiotensin II (Ang II), serotonin (5-HT) and potassium chloride (KCl) are expressed as EC_{50} (concentration that produces 50% maximal contraction). Data are presented as the mean value ± SEM of 10 to 15 experiments.
NTG = nitroglycerin.

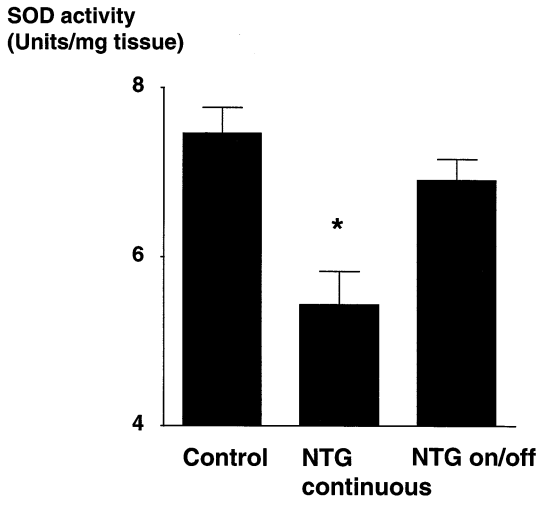

Figure 4. Effects of three-day NTG treatment of rabbits (continuous or 12 h patch on/patch off) on SOD activity in aortic homogenates. Continuous in vivo treatment with NTG significantly decreased total vascular SOD activity. This decrease was prevented by intermittent treatment with NTG. Data are presented as the mean values ± SEM of six to eight separate experiments. *$p < 0.05$ vs. control.

mal studies have shown that long-term treatment with NTG patches or NTG infusions leads to endothelial dysfunction. This was associated with increased vascular nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-mediated $O_2^{\bullet-}$ production (10), decreased activity and expression of the $O_2^{\bullet-}$-scavenging enzyme Cu/Zn SOD (11) and increased sensitivity of the tolerant vasculature to vasoconstrictors such as angiotensin II, phenylephrine and serotonin (13). The present findings confirm and extend these observations. Treatment of rabbits with NTG for three days caused a significant shift to the left of the concentration–response curve for angiotensin II, phenylephrine, serotonin and KCl. In addition, there was a significant increase in vascular $O_2^{\bullet-}$ generation (as determined with LDCL) and a decreases in total vascular SOD activity and Cu/Zn SOD expression. In organ chamber experiments with isolated aortic rings, we also established attenuation of the responses of the tolerant vasculature to the endothelium-dependent vasodilator ACh. All these findings may contribute, at least in part, to the marked decrease in sensitivity of the vasculature to NTG in response to continuous treatment.

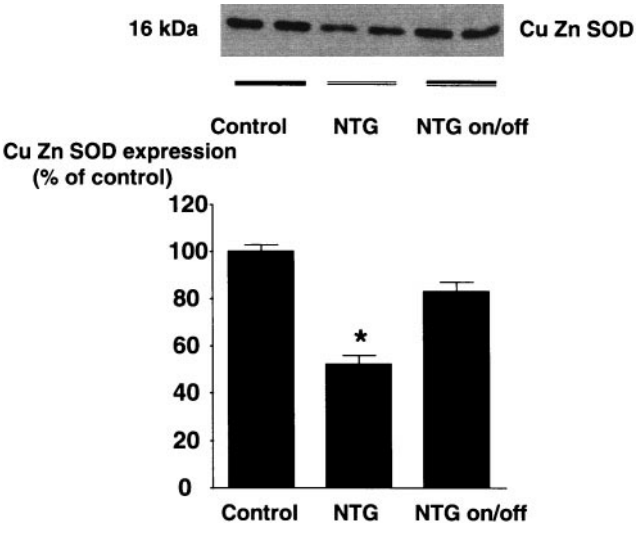

Figure 5. Upper panel, Representative Western blot illustrating the effects of three-day NTG treatment of rabbits (continuous or 12 h patch on/patch off) on the expression of Cu/Zn SOD in aortic homogenates. **Lower panel,** Densitometric analyses of four similar Western blots. Continuous in vivo treatment with NTG for three days caused a significant decrease in protein expression of Cu/Zn SOD, which was almost completely normalized by the intermittent treatment. *$p < 0.05$ vs. control.

Nitrate-free interval and vascular superoxide production. Many strategies have been suggested to prevent the phenomenon of nitrate tolerance, but the only approach that has gained clinical acceptance is a nitrate-free interval. Continuous intravenous (17,18), oral (19) or transdermal (20) treatment with NTG patches causes considerable tolerance to the preload effects of NTG, to its coronary vasodilator effects and to the ischemia threshold in patients with stable angina or myocardial infraction. These phenomena were prevented by a nitrate-free interval ranging from 8 to 12 h. Using 12-h path-on/patch-off NTG treatment, we observed that the sensitivity of the vasculature to NTG was markedly improved, $O_2^{\bullet-}$ production was reduced and endothelial dysfunction (as assessed by the vascular response to ACh) was almost completely normalized. The current study also demonstrates that a nitrate-free interval increases total vascular SOD activity as well as Cu/Zn SOD expression, as compared with values obtained from the vessels of animals receiving continuous NTG treatment.

An interesting observation of the present study was the LDCL response of the vasculature to the NOS inhibitor L-NMA. Incubation of control vessels with the NOS inhibitor markedly increased LDCL, indicating that endothelial-derived NO quenches LDCL under basal conditions. Vessels from NTG-treated animals, however, produced a markedly stronger basal LDCL signal, which was reduced by L-NMA (Fig. 3). These data go along with preliminary data where NTG infusion in rats increased NOS III expression and vascular $O_2^{\bullet-}$ production, which was blocked by preincubation of tolerant tissue with the NOS III inhibitor N^G-nitro-L-arginine (21). Therefore, long-term nitrate therapy may cause an uncoupling of NOS III, resulting in NOS III–mediated $O_2^{\bullet-}$ production. The vessels from animals treated intermittently with NTG showed a L-NMA response, similar to control vessels, suggesting that a nitrate-free interval of 12 h is sufficient to prevent the uncoupling of NOS III.

Nitrate-free interval, rebound angina and enhanced sensitivity to vasoconstrictors. Although a nitrate-free interval may restore the sensitivity of arteries and veins to NTG, several studies have demonstrated that patients with stable coronary artery disease treated with intermittent transdermal nitrate therapy experienced adverse effects for up to 12 h during the drug-free period. Rebound effects after nitrate withdrawal have first been described in ammunition workers

exposed to high concentrations of NTG. They showed a higher incidence of angina, myocardial infarction and sudden cardiac death on the nitrate-free weekends (22). These conditions may differ from the removal of NTG patches, but two earlier studies in patients with stable coronary artery disease suggest the existence of rebound angina during the nitrate-free interval (23,24). In these studies, NTG patches were applied for two continuous days, followed by a two-day nitrate-free period. Interestingly, patients consistently showed an increase in anginal attacks and sublingual NTG use on the first day after patch removal.

These observations were confirmed in a more recent study by Ferratini et al. (25) with transdermal NTG, where increased nocturnal angina was reported during the NTG patch-off period. In a multicenter trial, De Mots et al. (26) reported that 9 of 138 patients treated intermittently with NTG patches experienced a significant increase in rest angina during the NTG-free period. Patients receiving placebo showed no change in rest angina. Intermittent therapy with NTG patches has also been shown to alter the diurnal pattern of ischemia, with a loss of overnight nadir and a decrease in the anginal threshold for 4 to 6 h after patch removal, all of which suggest rebound ischemia (5). Results from the Second Transdermal Intermittent Dosing Evaluation Study (TIDES-II) also showed an increase in ischemia frequency during patch-off hours after use of low dose intermittent transdermal NTG (7).

Recent experimental as well as clinical studies have provided insight into the potential mechanisms underlying these rebound phenomena. Abrupt cessation of intravenous NTG therapy in chronically instrumented dogs induced rebound constrictions of the large coronary arteries (27). In these studies, large coronary artery constriction occurred, even in the absence of an activated circulating renin-angiotensin system, indicating that long-term NTG therapy may induce intrinsic abnormalities of the vasculature itself, such as changes in vasoconstrictor sensitivity. Also, in patients treated with NTG, removal of the patches was associated with a significant rebound constriction of the large coronary arteries and a substantial constrictor response to the endothelium-dependent vasodilator ACh (28). Acetylcholine induces relaxation by stimulating the release of NO from endothelial cells, but also stimulates muscarinic M_3 receptors on vascular smooth muscle cells, thereby inducing vasoconstriction. It remains to be determined whether an increase in ACh-induced coronary artery constriction reflects endothelial dysfunction or an increased sensitivity of the smooth muscle cells to vasoconstrictor agents (28).

The most striking observation of the present studies was that a 12-h nitrate-free interval failed to correct the hypersensitivity of the vasculature to agonists such as serotonin and phenylephrine. Serotonin-induced contractions of aortas from animals treated with a nitrate-free interval were enhanced, as compared with constrictions produced in nitrate-tolerant tissue. Although the precise mechanisms of NTG-induced hypersensitivity to vasoconstrictors remain to be established, more recent experimental studies indicate that the second messenger protein kinase C may play an important role in mediating this phenomenon. The increased vasoconstriction to different agonists in the setting of tolerance is inhibited in vitro by protein kinase C inhibitors (13). In addition, in vivo treatment with the protein kinase C inhibitor N-benzoyl-staurosporin has been shown to prevent NTG-induced vascular hypersensitivity to thromboxane mimetics and norepinephrine (29).

Study limitations. The dosage of transdermal NTG used in the present study was 0.4 mg/h. Assuming a uniform release of the drug, the NTG delivery rate in a 3- to 4-kg rabbit would average 2 to 3 μg/kg per min. The hemodynamic changes seen in response to this NTG concentration include reflex tachycardia (30,31), but no changes with respect to mean arterial pressure and hind-limb resistance (30). This particular NTG concentration is not uncommonly used in the treatment of patients with unstable angina or congestive heart failure (when given intravenously), and may therefore be achieved when nitrates are used long term. It is difficult to extrapolate the relevance of drug doses between different species, especially when body surface areas are very different; however, at the very least, one can conclude that our study may have implications with respect to intravenous administration and treatment with high doses of NTG in patients with congestive heart failure.

Clinical implications and conclusions. The present observations suggest that in our experimental model of nitrate tolerance, a nitrate-free interval almost completely restores nitrate sensitivity, reduces oxidative stress, normalizes SOD activity and Cu/Zn SOD expression, prevents the uncoupling of endothelial NOS and therefore corrects endothelial dysfunction. However, this nitrate-free period fails to prevent the development of hypersensitivity to vasoconstrictors, such as serotonin and phenylephrine. These observations may indicate that NTG-induced sensitization to vasoconstrictors, rather than decreased sensitivity of the vasculature to NTG, may account for the rebound phenomena observed after NTG withdrawal in intermittent therapy. Because concomitant treatment with beta-blockers (32) or angiotensin-converting enzyme inhibitors, or both, has been shown to prevent rebound phenomena in clinical (33) and experimental studies (27), the present findings encourage the standard practice of treating patients with stable angina with a combination of nitrates and beta-blockers and/or angiotensin-converting enzyme inhibitors.

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